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A Paracrine Paradigm for *in Vivo* Gene Therapy in the Central Nervous System: Treatment of Chronic Pain

ALAN A. FINEGOLD,[1,*] ANDREW J. MANNES,[2] and MICHAEL J. IADAROLA[1]

ABSTRACT

A limitation of current gene therapy efforts aimed at central nervous system disorders concerns distribution of vectors on direct injection into neural tissue. Here we have circumvented this problem by transferring genes to the meninges surrounding the spinal cord, achieving an *in vivo* gene transfer paradigm for treating chronic pain. The therapeutic vector consisted of a recombinant adenovirus encoding a secreted form of the potent endogenous opioid β-endorphin. In an inflammation model of persistent pain, administration of the vector into the cerebrospinal fluid (CSF) surrounding the spinal cord transduced meningeal pia mater cells. The resulting increase in β-endorphin secretion attenuated inflammatory hyperalgesia, yet had no effect on basal nociceptive responses. This demonstration of a gene transfer approach to pain treatment can be generalized to neurodegenerative disorcers in which broad spatial distribution of therapeutic effect is critical.

OVERVIEW SUMMARY

One of the underappreciated aspects that limits viral gene therapy in the CNS concerns inadequate tissue distribution of viral vectors. A novel approach is presented, in which a therapeutic product is delivered according to a meningeal-paracrine paradigm. As a test case, adenoviral delivery of a β-endorphin gene to meningeal cells surrounding the spinal cord specifically reduced chronic pain behavior in a rat model. In terms of pain control at the spinal level, this approach to pain gene therapy is easily applied as an outpatient therapy by lumbar puncture. The clinical utility of this meningeal-paracrine approach is broadly applicable to other CNS disorders in which large areas of brain tissue and/or large numbers of distributed neurons must be affected, such as Parkinson's or Alzheimer's disease, or amyotrophic lateral sclerosis.

INTRODUCTION

CONTROL OF CHRONIC PAIN is a worldwide public health problem (Bonica, 1990; Wall and Melzack 1994; Gureje *et al.*, 1998). Many disease processes and traumatic injuries can lead to a lifetime of unremitting severe pain. Current treatments often fall short of therapeutic goals and can eventually involve procedures that are invasive or associated with unacceptable side effects. In neuropathic pain after nerve injury, pain is poorly controlled by currently available methods. In cancer pain, intravenous or oral morphine is only partially effective, and is accompanied by debilitating side effects such as constipation, sedation, and respiratory depression. Furthermore, its practical use is often limited by concerns about addictive properties (Bonica, 1990; Wall and Melzack, 1994; American Pain Society, 1995; Bernabei *et al.*, 1998; Gureje *et al.*, 1998; Ingham and Foley, 1998). Attempts to localize delivery of opiates in order to reduce side effects include both mechanical and cellular approaches. Catheters can be implanted into the subarachnoid space and connected to infusion pumps, but are costly, difficult to maintain, and present an infection risk. Spinal grafts of clonal cell lines (Wu *et al.*, 1994; Beutler *et al.*, 1995) or xenografts of primary tissue (Sagen, 1998) have also been proposed, but are constrained by issues of control, maintenance, and invasive surgery.

One way to circumvent such issues and provide long-term pain control is to directly modulate pain through *in vivo* gene transfer (Iadarola *et al.*, 1997; Jacoby *et al.*, 1997; Anderson, 1998). In this article we focus on gene transfer intervention in

[1]Pain and Neurosensory Mechanisms Branch, NIH-NIDCR, Bethesda, MD 20892.
[2]Department of Anesthesiology, University of Pennsylvania, Philadelphia, PA 19104.
*Current address: Receptor Biology, Inc., 10,000 Virginia Manor Road, Suite 360, Beltsville, MD 20705.

the spinal cord, because pain from a broad spectrum of disorders can be controlled at this level of the central nervous system. Previous studies on direct intraparenchymal injections of adenoviral vectors (Ad-lacZ) expressing a *lacZ* transgene resulted in nonuniform distribution of transgene expression and limited spread of expression in neural tissue (Mannes *et al.*, 1998). This is a general problem in gene delivery to the central nervous system, and in particular is a problem in pain treatment. Since primary afferent nociceptive (pain) neurons from a given region of peripheral tissue innervate several spinal segments, multiple injections are required to achieve a significant anti-pain effect.

In contrast to intraspinal injections, intrathecal delivery of Ad-lacZ into the cerebrospinal fluid (CSF) surrounding the spinal cord did not yield viral access to cells of the spinal cord itself, but rather to cells of the pia mater, the meningeal layer directly apposed to the spinal cord (Mannes *et al.*, 1998). The pia mater thus provided a highly accessible target tissue for gene transfer using a meningeal-paracrine secretory approach. β-Endorphin is a neuropeptide with known pain-suppressing properties in humans and animals (Yaksh and Henry, 1978; Oyama *et al.*, 1980; Max *et al.*, 1985). Secretion of β-endorphin peptide from nonneural cells is efficiently directed by addition of a heterologous prepro-sequence from the nerve growth factor gene (Beutler *et al.*, 1995). The approach in the present report integrated these three sets of observations for therapeutic delivery of β-endorphin to the spinal cord via the pia mater. Given the ease of access to the pia mater, our strategy for functional intervention was to control neuronal excitability by local secretion of a neuromodulatory peptide, β-endorphin.

MATERIALS AND METHODS

Vector construction

A fusion gene (Beutler *et al.*, 1995) consisting of the nerve growth factor prepro-sequence followed by the β-endorphin peptide was ligated into the *Eco*RI–*Bam*HI restriction sites of pACCMV.pLpA (Becker *et al.*, 1994). The resulting recombinant adenoviral vector (first-generation, replication-deficient Ad5) containing the expression cassette was concentrated and purified. The titer of the final viral preparation was determined by absorption spectroscopy for particle number (5×10^{11}/ml)

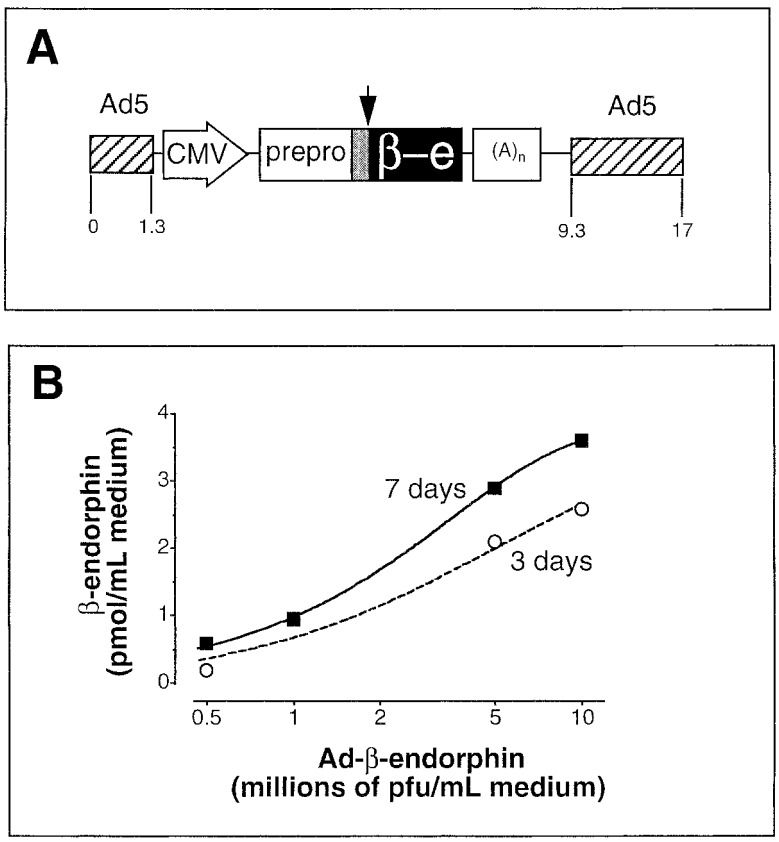

FIG. 1. Adenoviral vector-directed expression of secreted β-endorphin from cells cultured *in vitro*. **(A)** Structure of Ad-β-endorphin vector. An Ad5 recombinant (adenovirus sequences are hatched, map units indicated) expresses a fusion between a nerve growth factor prepro-sequence and β-endorphin (β-e), under control of the cytomegalovirus promoter (open arrow) and polyadenylation sequences $(A)_n$. Sequences (gray) directing cleavage by prohormone convertases (arrow) bridge the fusion gene. **(B)** Production of β-endorphin by bovine smooth muscle cells transduced by Ad-β-endorphin. Medium from cells incubated with indicated concentrations of virus was assayed for β-endorphin by radioimmunoassay. β-Endorphin levels at 3 days (open circles, dotted line) and 7 days (filled squares, solid line) postinoculation are plotted and superimposed by curves fitted to hyperbolae.

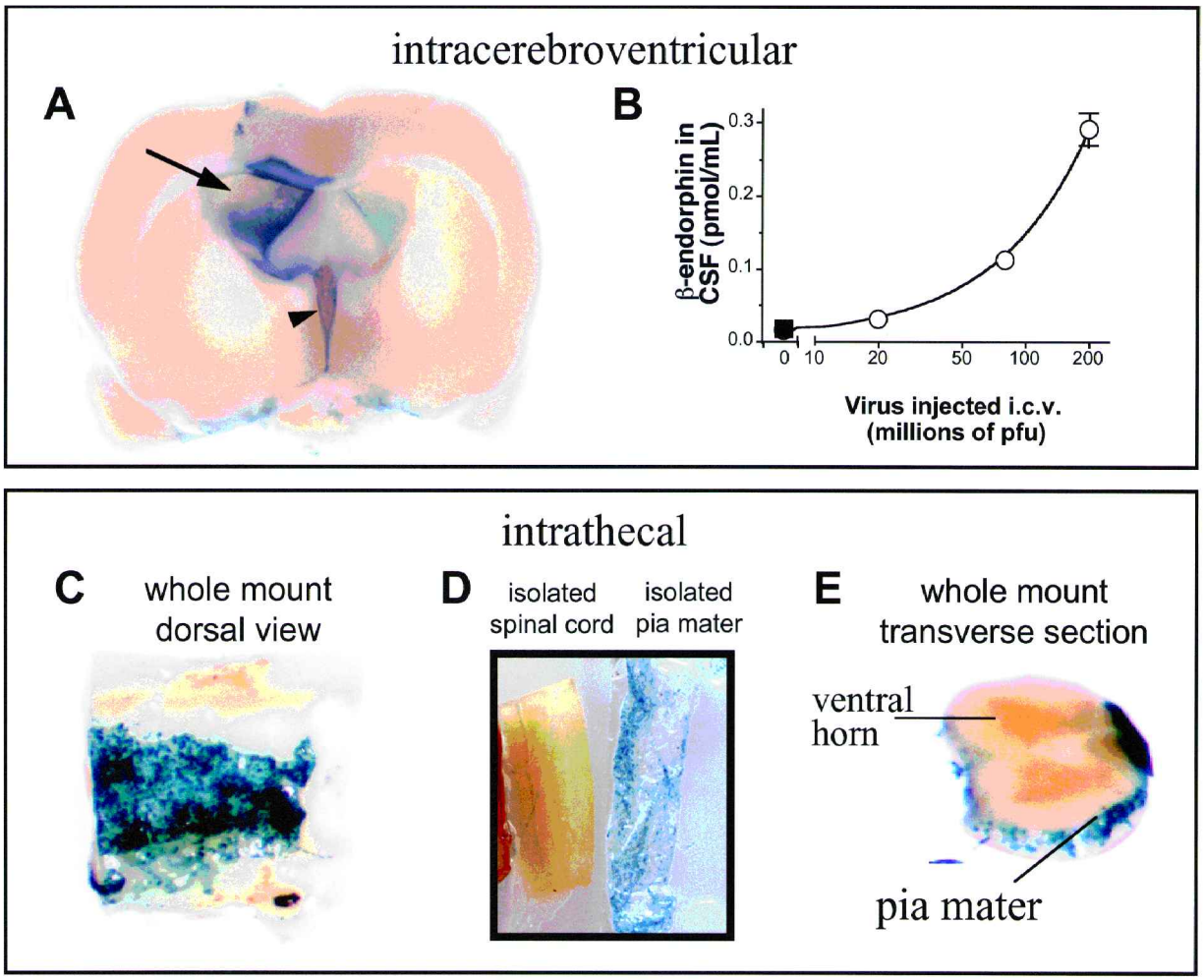

FIG. 2. *In vivo* gene transfer and production of β-endorphin. **(A)** X-Gal histochemistry of a coronal section of a rat brain 2 days after injection of Ad-lacZ and Ad-β-endorphin into the lateral ventricle. Note the blue product in ependymal cells of the lateral (arrow) and third (arrowhead) cerebral ventricles. Some staining of the needle track is evident in the cortex. **(B)** Dose response of Ad-β-endorphin *in vivo* (open circles). Various amounts of Ad-β-endorphin injected intracerebroventricularly (i.c.v.) directed secretion of β-endorphin, which was measured in CSF collected 2 days postinjection. Levels of β-endorphin in CSF from naive rats are indicated by a filled square. **(C–E)** Intrathecal administration of Ad-lacZ. At 3 days postinjection, spinal cords stained with X-Gal were viewed in three ways: **(C)** whole-mount view of dorsal aspect of spinal cord with intact pia mater; **(D)** after separation of the pia (blue stain, right) from the spinal cord itself (no stain, left); and **(E)** transverse section, tilted to visualize both the blue pia expression and the lack of stain in the parenchyma of the spinal cord. See **(C)** for β-endorphin secretion into CSF after intrathecal injection of Ad-β-endorphin.

or infection of HEK293 cells (4×10^9 plaque-forming units [PFU]/ml).

Cell culture

Bovine smooth muscle cells (AG08595A; Coriel Institute, Camden, NJ) were grown to confluence (3.8×10^6 cells per well) in 11-mm dishes, with daily changes of medium. Various concentrations of Ad-β-endorphin were added to wells and the medium was sampled on days 3 and 7 postinoculation.

β-Endorphin radioimmunoassay

β-Endorphin levels in the cell culture medium and rat CSF were determined by radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA). For sampling of rat CSF, a 27-gauge hypodermic needle attached to a syringe with no hub or a clear hub (essential for visualization of CSF withdrawn) was inserted into the cisterna magna and approximately 100 μl of CSF was removed. CSF or cell culture medium was boiled in water, clarified by centrifugation (12,000 $\times$ *g*, 5 min), and stored frozen at -70°C until assay.

Histological methods

After perfusion with 4% paraformaldehyde (but no postperfusion fixation), reaction of 2-mm-thick coronal slices of brains or sections of spinal cord with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, pH 7.3, 1 mg/ml at 37°C (Shimohama *et al.*, 1989) demonstrated a blue reaction product within 15 min, which was complete by 2 hr.

In vivo methods

Animal experiments were performed in accordance with National Institutes of Health Animal Care and Use Committee guidelines. For intracerebroventricular injections, virus stock solution was diluted into 50 m*M* Tris (pH 7.4), 10% glycerol, 10 m*M* $MgCl_2$ and infused into the left lateral cerebral ventricle (10 μl over 10 min). The same viral solution was infused intrathecally (10 μl over 2 min) through a PE-10 catheter. The catheter was inserted through an incision in the cisterna magna and advanced 3 cm, to approximate the rostral extent of the spinal cord segments innervated by the hindpaw (the peripheral site tested for nociceptive responses). Inflammation was induced by subcutaneous injection of 2 mg of carrageenan (type IV, C-3889; Sigma, St. Louis, MO) in a volume of 0.15 ml of phosphate-buffered saline (PBS) into the plantar surface of one hindpaw (200 to 300-g male Sprague-Dawley rats). After inflammation, there was no difference in edema (paw thickness) between Ad-β-endorphin rats and controls (both increased by approximately twofold). A radiant thermal test in unrestrained rats (Hargreaves *et al.*, 1988) was used to assess nociceptive responses by paw withdrawal latency. The observer was blinded as to the virus (control or Ad-β-endorphin) injected.

RESULTS

Secretion of β-endorphin in vitro and in vivo

Transgene expression from a recombinant adenoviral vector, named Ad-β-endorphin (Fig. 1A), was controlled by a constitutive viral promoter. Secretion of β-endorphin from cells cultured *in vivo* with Ad-β-endorphin increased with time and vector concentration from a minimum of 0.004 pmol/ml medium (0.5 million PFU/ml medium at 3 days) to a maximum of 3.5 pmol/ml medium per day at 7 days postinoculation (Fig. 1B), suggesting that such secretion might be sufficient to affect neuronal function *in vivo*.

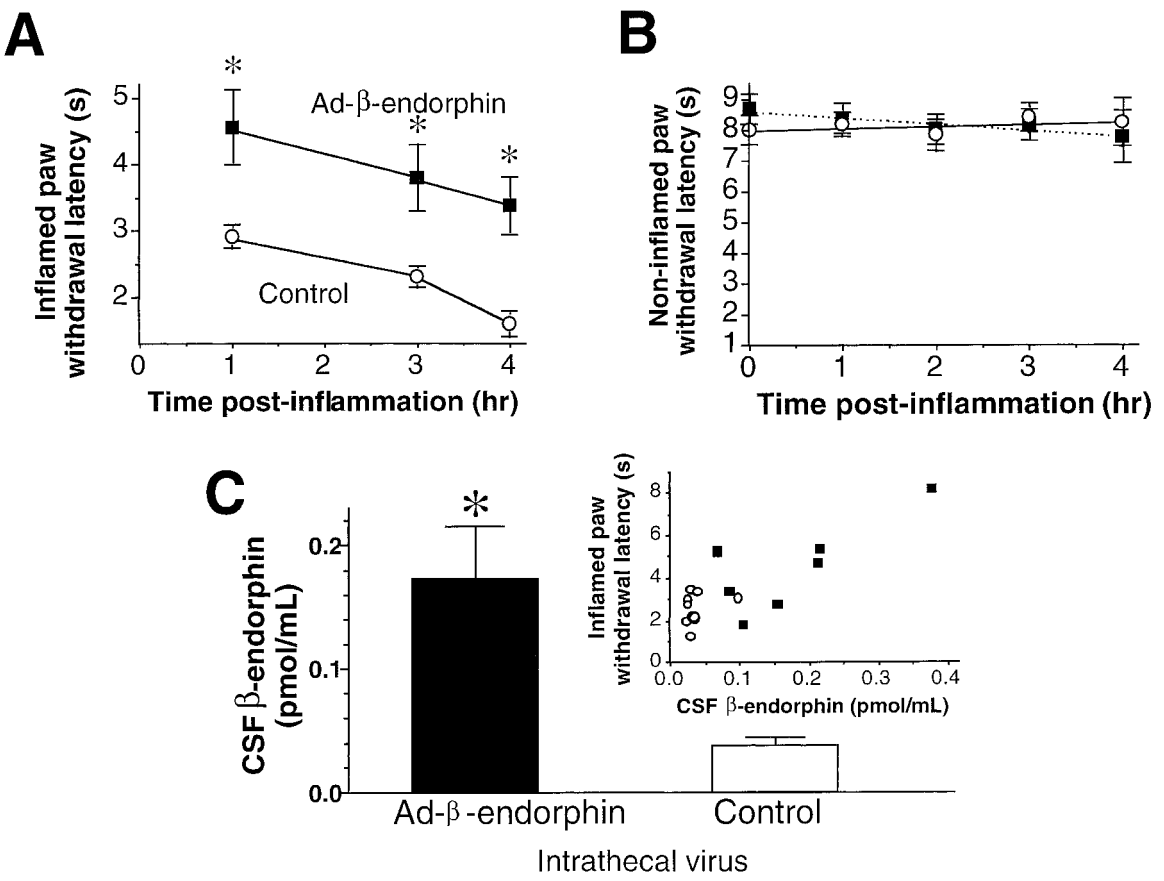

FIG. 3. Nociceptive behavior of rats in a carrageenan model of inflammatory pain. Three days prior to the pain test, rats were infused intrathecally over 2 min with 10^{10} particles of Ad-β-endorphin (filled squares) or Ad-lacZ (open circles), in a volume of 10 μl. **(A)** Attenuation of hyperalgesia by Ad-β-endorphin. Peripheral inflammation (Hargreaves *et al.*, 1988) induced hyperalgesia in one hind paw of rats injected intrathecally with Ad-lacZ. A significant prolongation of latencies in Ad-β-endorphin-treated rats was observed (data pooled from 12–27 animals, $p < 0.005$, two-tailed t test, indicated by asterisks) during the inflammation test period. **(B)** Noninflamed paw withdrawal latency after injection of Ad-β-endorphin. No significant difference between control and Ad-β-endorphin groups was observed (linear regression analysis). **(C)** β-Endorphin measurements from cisternal CSF of animals injected intrathecally with control or Ad-β-endorphin viruses. CSF was drawn 7 days postinjection to allow cisternal wound healing. *Inset:* A trend ($p < 0.08$) between increased paw withdrawal latencies and β-endorphin was observed after intrathecal administration of Ad-β-endorphin.

Introduction of the virus into the lateral ventricle of rats (intracerebroventricular injection) allowed determination of the ability of the virus to secrete β-endorphin after *in vivo* gene transfer to ependymal cells lining the cerebral ventricles. The extent of gene transfer to ependymal cells was visualized by coinjection of Ad-lacZ (Becker *et al.*, 1994) and detection of the transgene product by X-Gal histochemistry (Fig. 2A) and immunocytochemistry (not shown). Both techiques demonstrated cells strongly positive for the transgene product in the ependymal layer. This layer formed a barrier to virus penetration into brain, since little or no expression occurred in neurons or glia beyond the ependymal layer (Fig. 2A; noted also in Bajocchi *et al.*, 1993). Injection of Ad-β-endorphin produced a rapid, vector dose-dependent increase in CSF β-endorphin. As soon as 24 hr postinjection, the amount of β-endorphin secreted into the CSF reached 0.3 pmol/ml with 200 million PFU of virus, the maximum amount injected (Fig. 2B). Three days after intracerebroventricular injection, the level increased to 0.6 pmol/ml. These levels represent a 10- to 20-fold increase over normal β-endorphin levels in human or rat CSF, 0.01–0.03 pmol/ml (Kiser *et al.*, 1983; Nakao *et al.*, 1980).

Intrathecal injections rostral to the lumbar spinal cord were used to assess viral spread and transgene expression. Three days after intrathecal Ad-lacZ injection, the spinal cord was removed with pia mater attached and stained for transgene expression. Expression was observed in cells of the pia mater (Fig. 2C–E) but not of the spinal cord itself (Fig. 2D and E), evident after removal of the pia mater (Fig. 2D) or transverse section (Fig. 2E). The expression spread 2–3 cm in the rostral–caudal dimension. In animals injected intrathecally with Ad-β-endorphin, CSF β-endorphin content, sampled from the cisterna magna, was 0.2 pmol/ml at 7 days and returned to endogenous levels (0.03 pmol/ml) by 15 days. The cisterna magna is anterior to the catheter tip and may not be the optimal site for sampling secretion from spinal pia mater, and thus these data may represent an underestimate of β-endorphin secretion. Data also suggest that transgene expression peaked between 3 and 7 days and waned after 15 days, a time course similar to that observed by other investigators (Bajocchi *et al.*, 1993; Mannes *et al.*, 1998). These data demonstrate secretion from both ependymal and pia mater cells.

Effect of β-endorphin on pain in rats

On the basis of these data, the ability of Ad-β-endorphin to attenuate nociceptive (pain) responses in the peripheral inflammation model was examined following gene transfer to the spinal pia mater. To control for nonspecific effects of the viral vector, Ad-lacZ was injected intrathecally. Baseline thermal nocicpetive tests in unrestrained rats prior to inflammation indicated that paw withdrawal latencies to a radiant thermal stimulus were 8.7 ± 2.6 sec for Ad-lacZ, and 8.9 ± 2.5 sec for Ad-β-endorphin (mean ± standard error; $N = 33$ for each virus). In the control group, inflammation of one hind paw produced a marked decrease in withdrawal latency (e.g., to 2.3 ± 0.7 sec at 3 hr postinflammation), an indication of hyperalgesia. Injection of Ad-β-endorphin significantly attenuated the hyperalgesic response at each time point tested (1, 3, and 4 hr; Fig. 3A). Because the primary afferent inputs are lateralized, the inflammation affects only one side of the rat and the contralateral, noninflamed, paw of these animals serves as an internal control for nonspecific or generalized effects on nociceptive responses. Latencies of this control paw remained constant during the period of behavioral testing for both control (Ad-lacZ) and Ad-β-endorphin groups (Fig. 3B). This indicated a lack of toxicity or nonspecific pharmacological effects from either virus, emphasizing that the effect of β-endorphin was observed only during neural activation by inflammation of the primary afferent.

Animals injected with Ad-β-endorphin exhibited signficantly higher levels of β-endorphin than did control Ad-lacZ animals (Fig. 3C; 0.17 ± 0.04 versus 0.037 ± 0.007 pmol/ml; $p < 0.007$ by t test). Although we were unable to obtain CSF from all animals tested, a trend in the degree of antihyperalgesia (measured 3 hr postinflammation) and increased β-endorphin levels was apparent in the Ad-β-endorphin group (Fig. 3C, inset; $r^2 = 0.42$, $p < 0.08$). These data provide further support for the hypothesis that secretion of the transgene product specifically affected hyperalgesic responses seen with persistent pain.

Administration of naloxone, a specific opioid antagonist, tested the hypothesis that the antihyperalgesic actions of Ad-β-endorphin were due to an opioid mechanism. For this experiment, two groups of 18 animals, one injected intrathecally with Ad-lacZ and the other with Ad-β-endorphin, were inflamed as in Fig. 3A with carrageenan for 3 hr. Thermal testing of these animals confirmed that the Ad-β-endorphin group was less hyperalgesic than the control group (Fig. 4). At this point, naloxone was delivered systemically. Determination of paw withdrawal latencies 40 min after naloxone revealed that the antihyperalgesic effect of Ad-β-endorphin was reversed ($p < 0.04$, one-tailed t test; Fig. 4). Thus, an opioid receptor mechanism attenuated the antihyperalgesia due to Ad-β-endorphin.

DISCUSSION

The method of gene transfer to nonneuronal cells to affect neuronal function, described here, is a highly accessible para-

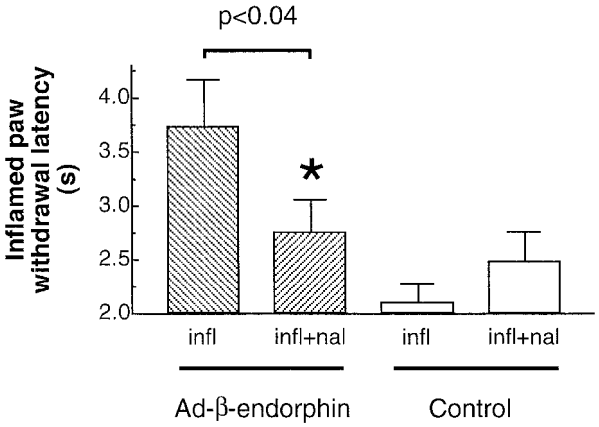

FIG. 4. Naloxone attenuation of Ad-β-endorphin-mediated anti-hyperalgesia. 3–4 hours post inflammation, naloxone (2 mg/kg, intraperitoneal) was injected and latencies re-measured after an additional 40 min to allow drug dispersal (N = 18).

digm for gene therapy approaches to central nervous system disorders. This paracrine paradigm has two key advantages for functional gene therapy. The first is the nonneuronal meningeal target, which provides for both a simple route of administration and allows manipulation of neurobehavioral function. The second key feature is spatial distribution of vector and secreted transgene product. Ad-lacZ spreads widely within the intrathecal space (Fig. 2D), demonstrating that the vector can distribute enough to modulate excitability over a multisegmental domain arising from primary afferent branching. This *in vivo* approach may need to be performed only once, with improvements in vector design for long-term, regulatable expression (Rivera *et al.*, 1996; A-Mohammadi and Hawkins, 1998; Hermens and Verhaagen, 1998; Saitoh *et al.*, 1998). This contrasts with intrathecal drug pumps, which require ongoing maintenance and pose an infection risk. Opioid genes may also be introduced into the peripheral nervous system, either directing secretion from primary afferent cells (Bras *et al.*, 1998), or directing local secretion from cells surrounding nociceptive nerve endings. β-Endorphin is effective against pain in models of knee inflammation and arthritis (Sluka and Westlund, 1993; Martinez *et al.*, 1996) and thus intraarticular injection of Ad-β-endorphin, like intraarticular morphine (Stein *et al.*, 1991), may be a therapeutically useful synovial-paracrine approach to gene therapy for arthritis (Jorgensen and Gay, 1998) analogous to the present meningeal-paracrine approach.

A surprising finding from these experiments on viral delivery of β-endorphin concerns the specificity of action toward different types of pain. Morphine alters baseline thermal sensation at the same doses that affect inflammation-induced hyperalgesia (Ho *et al.*, 1997; Hargreaves *et al.*, 1988), i.e., in rats, morphine is analgesic. In contrast, the reduction in hyperalgesia observed with Ad-β-endorphin was not accompanied by changes in baseline nociception, measured in the contralateral paw. Other opiate agonists such as bremazocine and GR89,696 also share this selectivity (Ho *et al.*, 1997), suggesting that the virally delivered peptide was acting at a similar opioid-sensitive site specifically related to hyperalgesia. Binding and functional data indicate that β-endorphin, bremazocine, and GR89,696 all recognize a non-μ, non-δ, non-κ_1 site in the spinal cord, termed κ_2 (Ho *et al.*, 1997) or ε (Nock *et al.*, 1993). κ_2/ε sites exist in the spinal cords of a wide variety of species including humans (Caudle *et al.*, 1998), suggesting that the antihyperalgesic-specific effects of β-endorphin observed in this study may predict efficacy in chronic pain syndromes in humans.

The simplicity of this meningeal-paracrine gene therapy approach, rapidity of expression, ease of application, and apparent lack of side effects offer the possibility of a more general clinical utilization, especially in spinal cord injury or neurodegenerative diseases. Substitution of the β-endorphin sequence for growth factor genes (Barkats *et al.*, 1998; Tuszynski *et al.*, 1998) provides a means of supplying crucial factors to the spinal cord for increasing motor neuron survival in diseases such as spinal muscular atrophy or amyotrophic lateral sclerosis (Zurn *et al.*, 1998), or following spinal injury. Our results demonstrate *in vivo* delivery of the neuropeptide β-endorphin from a nonneuronal target cell, which then attenuates persistent pain behavior. This approach forms the basis for a novel therapy for pain control, which can be extended to other neurobiological disorders.

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Address reprint requests to:
Dr. Michael J. Iadarola
National Institutes of Health
National Institute of Dental and Craniosacral Research
Pain and Neurosensory Mechanisms Branch
Building 49, Room 1A11, 49 Convent Drive, MSC 4410
Bethesda, MD 20892-4410

E-mail: Michael.Iadarola@nih.gov

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